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(54) Title: USE OF UROKINASE-TYPE PLASMINOGEN ACTIVATORS TO INHIBIT HIV INFECTIVITY

(57) Abstract

An *in vitro* method of inhibiting the infectivity of Human Immunodeficiency Virus (HIV) in a liquid that may contain HIV by exposing the liquid to a urokinase-type plasminogen activator at a concentration and for a time sufficient to inactivate HIV in the liquid.

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USE OF UROKINASE-TYPE PLASMINOGEN ACTIVATORS TO INHIBIT HIV INFECTIVITY

Background of the Invention

The invention relates to the use of urokinase-type plasminogen activators (u-PA) to inhibit the infectivity of human immunodeficiency viruses.

Human immunodeficiency virus (HIV), human T-cell lymphotropic virus III (HTLV-III), lymphadenopathy-associated virus (LAV), and AIDS-associated retrovirus (ARV) have been identified as the cause of AIDS. Popovic et al., Science, 224:497-500 (1984). One of the difficulties in preventing infection by these viruses is the extensive amino acid sequence variation, particularly in the envelope glycoprotein gp120, between different HIV variants, e.g., as described in Starcich, B.R. Cell, 45:637-648 (1986) and Hahn et al., Science, 232:1548-1553 (1986). Examples of these HIV variants include HIV-RF, Popovic et al., Science, 224:497-500 (1984), HIV-WMJ-1, Hahn B.H. et al., Science, 232:1548-1553 (1986), HIV-LAV, Wain-Hobson et al., Cell, 40:9-17 (1985), and ARV-2, Sanchez-Pescador et al., Science, 227:484-492 (1985).

In spite of the sequence variations, the different HIV variants include a so-called "principal neutralizing domain" (PND) or "V3 loop," which is located between the Cys residues at amino acid locations 296 and 331 of the envelope glycoprotein gp120 in HIV-IIIB (and corresponding amino acid locations in other HIV variants) following the amino acid numbering scheme for HIV variant HIV-IIIB (BH10) described in Ratner et al., Nature, 313:277-284 (1985). This numbering scheme requires a seven amino acid shift, because later studies showed a different starting amino acid for the envelope protein.

Thus, Ratner et al. imprecisely described these cysteine residues as being located at 303 and 338.

The PND or V3 loop was shown by LaRosa et al., Science, 249:932-935 (1990), to be conserved in more than 91% of 245 different HIV isolates analyzed. Consistent with the conserved nature of the V3 loop is the finding that HIV infectivity is dependent on its integrity. For example, Schulz et al., AIDS Res. Hum. Retrovir., 9:159-166 (1993), showed that mutation at Arg^{314} in the V3 loop dramatically reduced infectivity.

However, even the relatively conserved PND amino acid sequences of different HIV variants are highly varied. In spite of this variability, there is a high degree of conservation in the immunologically critical central region of the PND. Specifically, a Gly-Pro-Gly (GPG) sequence at the "tip" of the V3 loop occurs in over 90% of known variants. These three conserved amino acids occur at positions 312, 313, and 314 of the HIV envelope protein in HIV-IIIB (and at corresponding amino acid locations in other HIV variants). The sequence Gly-Pro-Gly-Arg (GPGR, SEQ ID NO:1) occurs in over 80% of known variants.

Although the V3 loop is important for viral entry into cells and syncytium formation, its exact role remains unclear. However, it has been suggested that the V3 loop interacts with a cellular surface proteinase that would either cleave it as a prerequisite for viral entry or act as a secondary binding site in the absence of cleavage. Antibodies that bind to the tip of the V3 loop and inhibit cleavage also neutralize the virus, which supports the theory that cleavage of this tip region is important for viral entry. Clements et al., AIDS Res. Hum. Retrovir., 7:3-16 (1991); Stephens et al., Nature, 343:219 (1990); and Meylan et al., AIDS, 6:128-130 (1991).

Urokinase-type plasminogen activators include urokinase (UK) in both low and high molecular weight forms. High molecular weight UK (HMW-UK, MW of 53 kDa) is a disulfide-linked dimer having a heavy (B) chain (amino acids 159-411) and a light (A) chain (amino acids 1-158). UK is a naturally occurring serine protease which is highly specific for plasminogen, and is thus an effective fibrinolytic agent. UK is well tolerated when injected intravenously, e.g., for thrombolytic therapy, at bolus dosages as high as 20 mg. Mathey et al., Am. J. Cardiol., 55:878 (1985).

Low molecular weight UK (LMW-UK) includes the entire B chain of UK plus a small portion of the A chain connected by a disulfide bond, and has a MW of about 33 kDA when measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis. LMW-UK is missing the UK receptor binding domain as described in Appella et al., J. Biol. Chem., 262:4437 (1993).

Summary of the Invention

The invention is based on the discovery that the major portion of the activation site loop of plasminogen is highly homologous, both in amino acid sequence and in three-dimensional structure, to the highly conserved sequence GPGR (SEQ ID NO:1) in the tip of the PND or V3 loop of the HIV-1 envelope protein gp120.

Furthermore, it was discovered that although urokinase-type plasminogen activators (u-PAs) are highly restricted enzymes whose principal substrate is plasminogen, these enzymes also inhibit HIV-1 infectivity, i.e., inhibit the infection of a cell by HIV-1, by cleaving the tip of the V3 loop immediately adjacent and downstream of the Arg residue (R) in the sequence GPGR (SEQ ID NO:1). This finding is in contrast

with the theory that cleavage of the gp120 envelope protein is required for viral entry into a cell.

The effect of the u-PA, e.g., UK, is time and concentration dependent, and is relatively specific to u-PAs, since other proteases such as tissue plasminogen activator (tPA), thrombin, or plasmin, did not inhibit HIV infectivity.

In general, the invention features an *in vitro* method of inhibiting the infectivity of HIV in a liquid, e.g., blood or a blood product, that may contain HIV, by exposing the liquid to a u-PA, e.g., HMW- or LMW-UK, or an active fragment of UK including the catalytic domain of the B chain of UK, at a concentration, e.g., 0.1 to 10.0 μM of u-PA in the liquid, and for a time, e.g., at least 15 minutes, sufficient to inactivate HIV in the liquid. The u-PA cleaves the envelope glycoprotein, gp120, of HIV between amino acids R and X in an amino acid sequence GPGRX (SEQ ID NO:2) in the V3 loop, wherein X is any amino acid, e.g., valine (V).

The method can include a further step of removing plasminogen from the liquid prior to exposing the liquid to the u-PA. As an additional step, the method can include returning the removed plasminogen to the liquid after the HIV has been inactivated by the u-PA. In all of these methods, the u-PA can be bound to a solid matrix, e.g., an agarose column.

The invention also features the use of a u-PA for the manufacture of a medicament for inhibiting the infectivity of HIV, the medicament including a gel, cream, or paste excipient and a u-PA, e.g., at a concentration of at least 2 to 20 μM in the excipient.

The invention further features a method of inhibiting the infectivity of HIV in a bodily fluid that may contain HIV by exposing the bodily fluid to the medicament of the invention at a concentration and for a

time sufficient to allow the medicament to inactivate HIV in the fluid.

In addition, the invention features the use of a u-PA for the manufacture of a medicament for inhibiting the infectivity of HIV in a patient. The medicament is administered to the patient in an amount and for a time sufficient to achieve a sustained blood concentration of the u-PA of 0.1 to 10.0 μM for at least 15 minutes, and preferably for more than an hour and up to several hours to days, either continuously, or at repeated intervals. This administration can include the further steps of removing the blood from the patient and removing plasminogen from the blood before contacting the blood with the u-PA, and optionally returning the removed plasminogen to the patient's blood. The plasminogen can be "removed" from the blood by plasmapheresis or with a plasmin inhibitor such as aprotinin or α_2-antiplasmin. Such a plasmin inhibitor can be administered by infusion in an amount to neutralize at least about 40 percent of the plasmin present in the plasma, e.g., by achieving a bloodstream concentration of about 0.2 mg/μl of the inhibitor.

As used herein, the term "urokinase-type plasminogen activator" ("u-PA") means any form of native or recombinant UK, or any native or recombinant fragment of full-sized (HMW) UK which contains at least the catalytic domain of the B chain, i.e., the full B chain with ten or more amino acids removed from the C-terminus, and which inhibits the infectivity of HIV-1, e.g., by cleaving the tip of the V3 loop of the gp120 envelope glycoprotein of HIV-1, with at least the same proteolytic efficiency as native UK, as determined by the assays described below. The term "u-PA" thus includes natural or recombinant forms of HMW-UK, low molecular weight UK (LMW-UK), and fragments that include the complete B-chain

(amino acids 159-411), or any catalytically active B-chain fragments. As used herein, the terms urokinase, "UK," and "HMW-UK" refer to the native or recombinant, full-sized form of the protease.

As used herein, an "HIV variant" is a particular strain of HIV or HIV-1 that has a distinct amino acid sequence for the envelope glycoprotein. HIV-1 variants include, for example, HIV-1IIIB, HIV-1RF, HIV-1MN, and HIV-1SC. The V3 loop amino acid sequence of the MN variant occurs in the majority of known HIV-1 strains. The RF variant sequence occurs in about 10 percent of known HIV-1 strains.

As used herein, to "inactivate HIV" means to inhibit or prevent the HIV from infecting a cell, e.g., by preventing the HIV from entering the cell.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described below. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

Other features and advantages of the invention will be apparent from the detailed description, and from the claims.

Description of the Drawings

Fig. 1 is a diagram representing the amino acid sequences of the urokinase binding site on the activation loop of plasminogen and of the V3 loop of HIV variants HIV-1RF, HIV-1MN, and HIV-1IIIB.

Fig. 2 is a bar graph showing the percent inhibition of HIV infectivity (protection) by UK on three different HIV-1 variants.

Fig. 3 is a bar graph showing the percent inhibition of HIV-1RF infectivity (protection) as a function of increasing UK concentrations.

Fig. 4 is a graph showing the time dependence of the inhibitory effect of UK on HIV-1RF infectivity.

Fig. 5 is a bar graph showing the inhibitory effect of UK and other proteases and inhibitors on HIV-1RF infectivity.

Fig. 6 is a graph showing dose-dependent suppression of viral reproductivity by high and low molecular weight UK in H-9 cells.

Fig. 7 is a graph showing suppression of viral reproductivity by HMW-UK in peripheral blood mononuclear cells.

Detailed Description

Urokinase-type plasminogen activators, e.g., HMW-UK and LMW-UK, are serine proteases whose principal substrate is plasminogen. HMW-UK can be prepared from pro-UK, e.g., derived from *E. coli* by standard techniques, and is available commercially, e.g., from Green Cross (Osaka, Japan).

As described below, LMW-UK has a less restricted substrate selectivity than HMW-UK, and was found to be five-fold more potent than HMW-UK in cleaving the V3 loop of HIV-1RF or HIV-1MN. LMW-UK is therefore better suited to inactivate a greater number of HIV-1 strains. LMW-UK is available commercially, e.g., under the name ABBOKINASE™ from Abbott Laboratories, Chicago, Illinois.

A recombinant form of UK that consists exclusively of the B chain (resfdues 159-411), preferably with the

cysteine at location 279 replaced by an other amino acid, e.g., alanine, to prevent dimerization of the molecule, is especially useful in the present invention. Recombinant B chain or active B chain fragments of UK can be made by standard techniques.

The enzyme binding or cleavage site for u-PAs, e.g., UK, in plasminogen consists of a loop of amino acids, which is represented as a linear amino acid sequence in Fig. 1. This cleavage site has the sequence CPGR**V**VGGC (SEQ ID NO:3), in which cleavage occurs between Arg^{560} and Val^{561} (shown in bold) to form plasmin. This plasminogen cleavage site loop was discovered to be very similar or homologous in amino acid sequence to the central region of the PND (V3 loop) of the gp120 envelope glycoprotein of HIV-1, which has been shown to be critical for HIV infections.

The spatial conformations of these two sequences were also found to be similar based on a published X-ray structure of the V3 loop of HIV-1MN as described in Ghiara et al., Science, 264:82 (1994), and the structure of the plasminogen loop was calculated using the computer program Quanta (Molecular Simulation).

The efficiency of the u-PA cleavage of the V3 loop depends, in part, on the viral amino acid located adjacent the Arg (R) residue of the GPGR sequence (SEQ ID NO:1). For example, the reaction for HMW-UK is most efficient for HIV-1 variants in which the adjacent amino acid is Val (V) such as in variant HIV-1RF, which has the PND sequence:

CTRPNNNTRKSITKGPGR**V**IYATGQIIGDIRKAHC (SEQ ID NO:4).

However, HMW-UK also cleaves the envelope proteins of other HIV variants, but effectively only at higher doses.

Fig. 1 also illustrates the amino acid sequences of the PNDs of two other HIV-1 variants, MN, and IIIB (SEQ ID NOS:5 and 6). As can be seen in the figure, the amino acid sequence of the central region of the PND of HIV-1RF is the most similar to the amino acid sequence of the plasminogen cleavage site loop, because it contains a Val residue adjacent to the Arg in the GPGR (SEQ ID NO:1) cleavage site sequence. Of the three variants studied, this sequence is most closely homologous to the activation site loop of plasminogen.

Even though this similarity between the HIV envelope protein and plasminogen amino acid sequences involves only three or four residues, applicants have discovered that this similarity is sufficient to allow u-PAs to bind to and cleave the V3 loop of the gp120 envelope glycoprotein of HIV-1 variants. As described in detail below, specific cleavage of the PND or V3 loop by UK, e.g., between GPGR (SEQ ID NO:1) and Val (V) in HIV-1RF, inhibited infectivity, whereas cleavage of the PND by thrombin has been said to have no effect on HIV infectivity. Clements et al., AIDS Res. Hum. Retrovir., 7:3 (1991).

Testing the Inhibitory Effect of u-PA

Cytotoxicity Assays

A slight modification of a standard MT-2 cell cytotoxicity assay was used as described in Pauwels, et al., J. Virol. Meth., 20:309 (1988). Briefly, serial dilutions of the antibody or serum were prepared in 50 µl volumes of complete medium and then 50 µl of a pre-diluted HIV stock was added to each well. After incubation for 1 hour at 37°C, 50 µl of a 4 x 10^5 MT-2 cell/ml suspension was added. The indicated concentration of antibody referred to the concentration present in the final 100 µl volume. The plates were

incubated for 5 days, at 37°C in 5% CO_2, then viable cells were measured using the metabolic conversion of the MTT formazan (1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan) dye powder (Sigma, St. Louis, MO) as described in Mosmann, T., J. Immunol. Meth., 65:55 (1983). 10 µl of a 5 mg/ml MTT formazan solution in PBS was added to each well.

After incubation at 37°C for 4 hours, the dye precipitate was dissolved by removing 50 µl of the cell supernatant, adding 65 µl of 10% Triton X-100 in acid isopropanol, and pipeting the samples up and down until the precipitate was dissolved. The optical density of the wells was determined at 540 nm with background subtraction at 690 nm. Percent inhibition was calculated by the formula: 1 - (virus control-experimental)/(virus control-medium control).

Two methods of exposing the virus to UK and cells were employed.

Method 1: HIV-1 virus (1:2 dilution, 2.7×10^{10} virus/ml) was incubated at 37°C with MT-2 cells (2×10^5/ml) in the absence or presence of a range of concentrations (0 to 8.0 µM) of HMW-UK, LMW-UK, or other test enzyme for 5 days. The cells and virus were centrifuged and washed daily in culture medium 1640 and fresh medium with enzyme was added. At the end of 5 days, the surviving cells were measured with MTT formazan as described above.

Method 2: HIV-1 virus was incubated with or without HMW-UK (2 µM) or other test enzyme for 15 to 60 minutes and then incubated with MT-2 cells (2×10^5/ml) for 5 days. No additional enzyme was added during the virus-cell incubation. Surviving cells were again measured by MTT.

In both of these methods, the percent inhibition of infectivity caused by the enzyme was calculated from

the control value determined from culturing virus plus MT-2 cells alone without the enzyme. All experiments were done in triplicate at least twice.

In one experiment, Method 1 was used to determine the effectiveness of 2.0 μM UK to inhibit infectivity of three HIV variants, HIV-1RF, HIV-1MN, and HIV-1IIIB (2.7 x 10^{10} virus/ml). As shown in Fig. 2, inhibition of infectivity was HIV variant dependant, with the HIV-1RF variant being the most sensitive to the inhibitory effect of UK (inhibition, i.e., protection, of almost 60% compared to control at 2 μM UK). This corresponds to the fact that the amino acid sequence of the PND of the HIV-1RF variant is also the most similar to the amino acid sequence of the plasminogen activation loop (see Fig. 1). HIV variants HIV-1MN and HIV-1IIIB were inhibited about 25% and 10%, respectively, compared to control at 2 μM UK. Greater inhibition was achieved at higher doses of HMW-UK or by using LMW-UK.

In another experiment, Method 2 was used to determine the inhibitory effect of UK on cell infectivity by HIV-RF. As shown in Fig. 3, the inhibitory effect was dose dependent, ranging from about 100% inhibition at 4.0 to 8.0 μM UK (columns 1 and 2) to about 18% at 0.02 μM UK (column 8). Columns 3 through 7 show the results of decreasing UK concentrations of 2.0, 1.0, 0.5, 0.2, and 0.1 μM, respectively.

The UK effect was also found to be time-dependent, reaching a plateau in about 45 to 60 minutes, as shown in Fig. 4. Using Method 2, HIV-RF was incubated with 2 μM UK (2.7 x 10^{10} virus/ml) for 15, 30, 45, and 60 minutes prior to the incubation of virus with MT-2 cells. As shown, the percent inhibition rose from about 40% at 15 minutes to over 70% at 45 minutes.

The Method 2 experiments illustrated in Fig. 5 indicate that the inhibitory effect of 2.0 μM of both

HMW-UK and LMW-UK is specific, catalytic, and directed against the HIV-1 itself, rather than the virus-cell complex. For example, lane 1 shows the results of incubating HMW-UK with HIV-RF for 1 hour, and then incubating this mixture with MT-2 cells ($2x10^5$/ml) for 5 days. In this test, the UK provided a 75% inhibition of infectivity. This result was unaffected by the addition of a specific, irreversible UK inhibitor, Glu-Gly-Arg chloromethylketone (GGAck, 20 μM for 30 minutes), one hour after exposure of UK to the virus (lane 2). In a similar experiment, the addition of GGAck did not affect the ability of LMW-UK to provide an inhibition of infectivity of about 70% (lane 8). These results suggest that the inhibition by UK is not CD4-related. Moreover, soluble CD4 was found to still bind to UK-treated virus.

These results also indicate that viral inactivation had occurred within the first hour, before the addition of GGAck. This conclusion is supported by the observation that when cells were exposed to virus for 4 hours prior to introduction of the 2.0 μM UK for 5 days, essentially no inhibition of infectivity took place (lane 3). Catalytic inactivation of UK by diisopropylflurophosphate (DFP) pretreatment also nullified the effect of UK (lane 7). Similarly, when thrombin (2.0 μM) was added to virus and cells along with UK for one hour, the thrombin cleaved the UK (thromb-UK) rendering it catalytically inactive (only 0.5% catalytic efficiency of UK). This cleaved form of UK did not inhibit virus infectivity (lane 4). However, since thromb-UK binds to plasminogen more tightly (7.5-fold) than UK, Liu et al., Blood, 81, 980 (1993), this finding supports the conclusion that catalysis rather than binding was responsible for the antiviral effect.

Other proteases like thrombin or tPA had little or no effect on viral infectivity (lanes 5 and 6). Thrombin

by itself had no significant effect on HIV infectivity against either HIV-1RF (lane 6) or HIV-1IIIB. Similarly, plasmin incubated for 1 hour had little effect (lane 9), and the plasmin inhibitor aprotinin (1,000 KIU, Miles Laboratories) did not attenuate the inhibitory effect of UK when incubated together for 1 hour.

Viral Titration Studies

Virus titration studies using H-9 cells and the p24 antigen assay were performed to evaluate the role of a cell surface receptor for UK discovered by receptor binding studies, which showed that H-9 cells, in contrast to the MT-2 cells, have a high affinity (k_D = 0.25 nM, B_{max} = 4.3 x 10^4 sites/cell; MW = 50,000) receptor for HMW-UK. This result is consistent with the well-established pro-urokinase/urokinase cell receptor found on monocytes, lymphocytes, and many other cells, e.g., as described in Vassalli et al., J. Cell Biol., 100:86 (1985). A similar u-PA receptor was also demonstrated on the virus itself by studies with radiolabeled HMW-UK. It is believed that this receptor was most likely derived from its mother cell.

For the p24 antigen assay, 2 x 10^5/ml H-9 cells were incubated with HIV-1RF (2.7 x 10^{10} virus/ml) in the presence or absence of various concentrations of HMW-UK (50 nM to 10 μM) or LMW-UK for 4 hours. Cells were washed and resuspended in 1 ml of growth media containing the same concentrations of UK, and incubated at 37°C. Cells were split at days 3 and 7 to 2 x 10^5/ml in media with corresponding UK concentrations. Supernatants were harvested at days 3, 5, 7, and 10, and the p24 antigen level was determined using the HIV-1 p24 Core Profile ELISA (DuPont-NEN) according to manufacturer's directions.

As shown in Fig. 6, HMW-UK provided a dose dependent suppression of viral reproductivity (■ control,

▲ UK (50 nM), ▲ UK (2 μM), and ■ UK (10 μM)). However, contrary to expectations, there was no promotion of this effect by the UK receptor since the most effective suppression occurred with 33 kDa LMW-UK, which is missing the receptor binding domain. In the presence of 2 μM LMW-UK, the p24 antigen level was only 4% of that present in the control (Fig. 6, ● LMW-UK (2 μM)). In the presence of 2 μM HMW-UK, the p24 level was 18% that of the control. Since LMW-UK does not bind to the u-PA cell receptor, these findings indicate that the cell receptor for UK does not promote the inhibitory effect of UK on HIV infectivity of H-9 cells. Similarly, blocking the UK receptor with 20 μM of DFP-treated UK had no effect on the UK's ability to inhibit HIV infectivity (Fig. 6, ▼ = 2 μM UK + 20 μM DFP-UK).

The apparent stronger inhibition of HIV-1RF by LMW-UK compared with HMW-UK is likely related to LMW-UK's less restricted substrate selectivity. When the proteolytic activities of HMW-UK and LMW-UK were compared, it was found that LMW-UK was two-fold more active against various Arg or Lys synthetic substrates (S2444, S2251, S2403, S2288) whereas the two enzymes were equivalent in their activation of plasminogen.

Syncytium Inhibition Assay

The assay method used was adapted from methods previously described in Hildreth et al., Science, 244:1075-1078 (1989). Briefly, 1 ml of HIV-1RF virus stock (2 x 10_7 virus particles/ml) was mixed with 5 x 10_5 CEMss (syncytia sensitive) cells in the presence or absence of LMW-UK at a final concentration of LMW-UK of either 2.0 or 10.0 μM in 2.0 ml of growth medium. The mixture was incubated at 37°C for 24 hours. The syncytia formation of these cells was observed under a microscope and recorded.

At 24 hours, the 10.0 μM concentration of LMW-UK prevented the formation of any syncytia (100% inhibition) compared to the control, in which large syncytia were present. The 2.0 μM concentration of LMW-UK also prevented the formation of syncytia compared to the control, but to a lesser extent (about 75% inhibition by a visual estimate).

Plasminogen Activation Assay

Plasminogen activation by UK (HMW or LMW) was measured in the presence of 1.5 mM S2251, a synthetic substrate for plasmin (H-D-Val-Leu-Lys-NH-phenyl-NO_2HCl), by measuring the absorbance (O.D.) increase of a reaction mixture over time at a selected wavelength 410 nm and at a reference wavelength 490 nm (410/490 nm) on a microtiter plate reader (Dynatech MR 5000, Dynatech Laboratories, Inc., Alexandria, VA). The reaction mixture contained S2251 (1.5 mM), Glu-plasminogen (2.0 μM) and HMW-UK (0.2 nM) or LMW-UK (0.2 nM). The reactants were mixed in 0.05 M sodium phosphate, 0.15 M NaCl, 0.2% BSA, 0.01% Tween-80, pH 7.4, and incubated at room temperature. The reaction rate was calculated in mini-absorbance per minute squared.

Hydrolysis of other synthetic substrates individually (S2444, Gly-Gly-Arg-NH-phenyl-NO_2HCl; S2403, Glu-Phe-Lys-NH-phenyl-NO_2; S2288, H-D-Ile-Pro-Arg-NH-phenyl-NO_2HCl; and S2302, H-D-Pro-Phe-Arg-NH-phenyl-NO_2HCl) was measured in the same way, but without plasminogen, so the reaction rate was calculated in mini-absorbance per minute. This assay can also be used to determine whether fragments of native UK, such as LMW-UK, activate plasminogen to a greater or lesser extent than native UK.

As shown in the table below, the results indicate that whereas HMW-UK and LMW-UK activate plasminogen at

comparable rates, LMW-UK is approximately two times as active against Arg or Lys synthetic substrates.

	Proteolytic Activity of UK					
Unit	$mA/min^2 \cdot nM$	$mA/min \cdot nM$				
Substrate	Glu-plasminogen	S2444	S2251	S2403	S2288	S2302
	2 μM	0.6 mM				
LMW-UK	1.065	2.150	0.006	0.047	0.868	0.010
HMW-UK	1.100	1.284	0.003	0.029	0.512	0.007

Human Peripheral Blood Mononuclear Cell Assay

A strong anti-HIV activity by UK (2.0 μM) was also observed with infection of human peripheral blood mononuclear cells (PBMCs) assay. This effect of UK on the reproduction of HIV-1RF in PBMCs was assayed as described in McLeod et al., Antimicro. Agents Chemother., 36:920-925 (1992). PBMCs were separated from human blood and stimulated with PHA and interleukin-2 for 3 days. The PBMCs were then incubated at 37°C with HIV-1RF ($2.7x10^{10}$ virus/ml) in the presence or absence of HMW-UK for 4 hours, washed 3 times and resuspended in 2 ml growth media with or without UK. The culture was refreshed with the same medium at days 3 and 7. The supernatant was harvested at days 3, 7, and 10 and assayed for P24 antigen as described above.

As shown in Fig. 7, after 7 days incubation, no viral protein was detectable in the UK-treated blood, in striking contrast to the untreated control (almost 400 pg/ml of p24). At day 10, the difference was even greater (zero compared to over 1800 pg/ml of p24).

Uses of Urokinase-Type Plasminogen Activators

Urokinase-type plasminogen activators described above can be used in a variety of ways to inhibit HIV infectivity both *in vitro* and *in vivo*. For example, u-

PAs can be bound to a solid matrix such as an agarose column, e.g., a SEPHAROSE™ column, and used to decontaminate any HIV-1 in blood or blood products, such as plasma, Factor VIII, or Factor IX. The following procedure was used to create such an agarose column. Ten grams of SEPHAROSE™ 4B gel was washed on a Buchner funnel in 3 volumes of coupling buffer (0.1 M phosphate buffer, pH 6.8). Excess supernatant was removed by gentle suction. Ten ml of UK (with a concentration 2 mg/ml) was dissolved in the coupling buffer and added to the gel. Sodium cyanoborohydride was added to a final concentration of 0.1 M and the suspension was agitated for 2 hours at room temperature. The gel was washed with 10 volumes of 1M NaCl. The unreacted aldehyde group was deactivated by agitating the coupled gel in 0.1 M ethanolamine, 0.1 M $NaCNBH_3$ at pH 6.8 for 2 hours at room temperature. After deactivation of the unreacted aldehyde group, the gel was washed with 1 M NaCl, followed by 0.1 M phosphate buffer, pH 7.0, containing 0.01 % sodium azide. The column is then ready for use to decontaminate blood and blood products of HIV.

Blood products containing plasminogen such as whole blood or plasma should have the plasminogen temporarily removed, e.g., by passage over Lysine-Sepharose (Sigma) by standard methods such as described in Castellino and Powell, Methods in Enzymology, 80:365-378 (1981), and then restored at the end of the decontaminating procedure, e.g., using the column described above. The same procedure described above for coupling UK to an agarose column can be used to couple lysine to such a column. On the other hand, plasminogen-free blood products such as Factor IX can be decontaminated of HIV-1 without the need for this temporary plasminogen removal.

In addition, u-PAs can be incorporated into medicaments such as vaginal gels or other lubricants and used to inactivate HIV-1 in bodily fluids such as semen or blood. The formulation and manufacture of such gels and lubricants are well known. High concentrations of u-PA are possible in the vaginal milieu in which plasminogen is absent. Therefore, the concentration of u-PA in such gels should be at least 2.0 to 20.0 μM, and can be greater depending on the excipient, e.g., 50 μM.

U-PAs, such as HMW-UK, LMW-UK, or the recombinant B chain of UK, can also be administered to a patient to inhibit the infectivity of HIV-1 in the patient. Compositions including u-PAs for therapeutic administration can be prepared by procedures well known in the art. For example, such compositions can be prepared as injectables, e.g., liquid solutions or suspensions. Solid forms for solution in, or suspension in, a liquid prior to injection also can be prepared. The u-PAs can be mixed with carriers or excipients that are pharmaceutically acceptable and compatible with the active ingredient. Examples of suitable excipients are water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof.

The preferred forms of u-PA for administration to a patient are LMW-UK or recombinant B chain of UK, e.g., in which the cysteine has been replaced by alanine, because like HMW-UK, these u-PAs have essentially no substrates other than plasminogen, but unlike HMW-UK, they do not bind to cell receptors, and are therefore less likely to induce other biological effects. Moreover, as discussed above, LMW-UK is more potent against a greater number of HIV-1 variants.

The u-PA compositions can be administered parenterally by conventional methods, e.g., by injection. For example, the compositions can be injected

intravascularly, e.g., intravenously or intra-arterially, subcutaneously, or intramuscularly. The compositions are administered in a manner compatible with the dosage formulation. The therapeutically effective quantity to be administered depends on the subject to be treated and the type or types of HIV-1 variants infecting the patient. Precise amounts of u-PAs required to be administered depend on the judgment of the practitioner and are specific for each individual. In particular, the u-PA compositions can be infused intravenously to achieve a steady bloodstream concentration of 0.1 to 2.0 μM LMW-UK or 0.5 to 10.0 μM HMW-UK. The concentration of u-PA, e.g., UK, in the bloodstream can be easily determined by a standard ELISA assay.

Infusions should be administered to achieve the desired bloodstream concentration of u-PA for at least 15 minutes, but an hour or more is preferred. In addition, bolus injections of 20 to 60 mg of u-PA can be administered at intervals to achieve the desired bloodstream concentration for an extended time period, e.g., at least one hour, and up to several hours or days.

When administered to patients at dosages required to inactivate HIV, e.g., an infusion of 20 to 100 mg/hour, u-PAs will induce systemic activation of plasminogen in the plasma, which may cause bleeding. However, this side effect can be avoided by removing the plasminogen, e.g., by plasmapheresis or by the simultaneous administration of specific plasmin inhibitors, prior to administration of the u-PA. In any event, extensive clinical experience with UK over more than 20 years indicates that it is well-tolerated and that once all the plasma plasminogen has been removed, the risk of bleeding is very low.

In a preferred administration scheme, the patient's blood is either cycled through a lysine-

SEPHAROSE™ column to temporarily remove the plasminogen via standard plasmapheresis techniques, or a plasmin inhibitor is administered to the patient by infusion prior to, or preferably during, initial u-PA administration in a dosage that neutralizes about 30 to 40 percent of the converted plasminogen. The remainder of the plasminogen is neutralized by α_2-antiplasmin that exists naturally in plasma. Therefore, after the first infusion of plasmin inhibitor, no further plasmin inhibitor is needed and UK can be infused alone as long as needed to inactivate HIV.

Suitable plasmin inhibitors include aprotinin (e.g., 100 IU/ml TRASYLOL®, Bayer, Leverkusen, Germany), α_1-antitrypsin, α_2-antiplasmin, α_2-macroglobulin, and monoclonal antibodies to plasmin.

Plasmin levels in blood or other fluids can be measured by various techniques. For example, as described in Salonen et al., Acta Ophthalmol, 65:3-12 (1987), the proteolytic activity of plasmin in fluids is measured by the radial caseinolysis procedure described in Saksela, Anal. Biochem., 111:276-282 (1981), using agarose gel and bovine milk casein as substrates. Human plasmin (20 casein units per mg; Kabi Diagnostica, Stockholm) is used as a standard. The results are expressed as micrograms of plasmin-like activity per ml of fluid. Plasmin levels can also be measured by various standard immunofluorescence techniques that can easily be adapted to detect plasmin in fluids.

Other Embodiments

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, that the foregoing description is

intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Jian-ning Liu
De-zhen Zhang
Victor Gurewich

(ii) TITLE OF INVENTION: USE OF UROKINASE-TYPE PLASMINOGEN ACTIVATORS TO INHIBIT HIV INFECTIVITY

(iii) NUMBER OF SEQUENCES: 6

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: WordPerfect (Version 5.1)

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: PCT/US95/----
(B) FILING DATE: November 1, 1995
(C) CLASSIFICATION:

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(A) APPLICATION NUMBER: 08/332,706
(B) FILING DATE: November 1, 1994
(C) CLASSIFICATION:

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(B) REGISTRATION NUMBER: 32,983
(C) REFERENCE/DOCKET NUMBER: 04547/014WO1

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(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Gly Pro Gly Arg
1

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(ix) FEATURE:
(E) OTHER INFORMATION: Xaa is any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Gly Pro Gly Arg Xaa
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 9
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Cys Pro Gly Arg Val Val Gly Gly Cys
1 5

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile Thr Lys Gly Pro
1 5 10 15

Gly Arg Val Ile Tyr Ala Thr Gly Gln Ile Ile Gly Asp Ile Arg Lys
20 25 30

Ala His Cys
35

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 35
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Cys Thr Arg Pro Asn Tyr Asn Lys Arg Lys Arg Ile His Ile Gly Pro
1 5 10 15

Gly Arg Ala Phe Tyr Thr Thr Lys Asn Ile Ile Gly Thr Ile Arg Gln
20 25 30

Ala His Cys
35

(2) INFORMATION FOR SEQUENCE IDENTIFICATION NUMBER: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 36
(B) TYPE: amino acid
(C) STRANDEDNESS: N/A
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile Arg Ile Gln Arg
1 5 10 15

Gly Pro Gly Arg Ala Phe Val Thr Ile Gly Lys Ile Gly Asn Met Arg
20 25 30

Gln Ala His Cys
35

What is claimed is:

1. An *in vitro* method of inhibiting the infectivity of Human Immunodeficiency Virus (HIV) in a liquid that may contain HIV comprising exposing the liquid to a urokinase-type plasminogen activator at a concentration and for a time sufficient to inactivate HIV in the liquid.

2. A method of claim 1, wherein said urokinase-type plasminogen activator is urokinase.

3. A method of claim 1, wherein said urokinase-type plasminogen activator is an active fragment of urokinase including the catalytic domain of the B chain of urokinase.

4. A method of claim 3, wherein said urokinase-type plasminogen activator is low molecular weight urokinase.

5. A method of claim 1, wherein said plasminogen activator cleaves the envelope glycoprotein, gp120, of HIV between amino acids R and X in an amino acid sequence GPGRX (SEQ ID NO:6) in the V3 loop, wherein X is any amino acid.

6. A method of claim 5, wherein amino acid X is valine (V).

7. A method of claim 1, wherein the concentration of said urokinase-type plasminogen activator in the liquid is 0.1 to 10.0 μM.

8. A method of claim 1, wherein the liquid is exposed to said urokinase-type plasminogen activator for at least 15 minutes.

9. A method of claim 1, wherein said liquid is blood or a blood product.

10. A method of claim 9, further comprising removing plasminogen from the liquid prior to exposing the liquid to said urokinase-type plasminogen activator.

11. A method of claim 10, further comprising returning the removed plasminogen to the liquid after the HIV has been inactivated by said urokinase-type plasminogen activator.

12. A method of claim 1, wherein said urokinase-type plasminogen activator is bound to a solid matrix.

13. The use of a urokinase-type plasminogen inhibitor for the manufacture of a medicament for inhibiting the infectivity of Human Immunodeficiency Virus (HIV), said medicament comprising a gel, cream, or paste excipient and the urokinase-type plasminogen activator.

14. The use of claim 13, wherein said plasminogen activator is present in said medicament at a concentration of at least 2 to 20 μM.

15. The use of claim 13, wherein said urokinase-type plasminogen activator is urokinase.

16. A method of inhibiting the infectivity of Human Immunodeficiency Virus (HIV) in a bodily fluid that may contain HIV comprising exposing the bodily fluid to a urokinase-type plasminogen activator (u-PA) at a concentration and for a time sufficient to allow the u-PA to inactivate HIV in the fluid.

17. The use of a urokinase-type plasminogen inhibitor (u-PA) for the manufacture of a medicament for inhibiting the infectivity of Human Immunodeficiency Virus (HIV) in a patient comprising administering to the patient a u-PA in an amount and for a time sufficient to achieve a sustained blood concentration of said u-PA of 0.1 to 10.0 μM for at least 15 minutes.

18. The use of claim 17, further comprising the steps of removing blood from the patient, and removing plasminogen from the blood before contacting the blood with said plasminogen activator.

19. The use of claim 18, wherein the plasminogen is removed from the blood with a plasmin inhibitor.

20. The use of claim 19, wherein the plasmin inhibitor is aprotinin or α_2-antiplasmin.

21. The use of claim 20, wherein the plasmin inhibitor is administered by infusion in an amount to neutralize about 40 percent of the plasmin present in the plasma.

Fig. 1

Cleavage Site for Urokinase

CPGR-VVGGC Activation Loop of Plasminogen (SEQ ID NO:3)

CTRPNNNTRKSITK..GPGR-VTYATGQIIGDIRKAHC HIV-1RF (SEQ ID NO:4)

CTRPNYNKRKRIHI..GPGR-AFYTTKNIIGTIRQAHC HIV-1MN (SEQ ID NO:5)

CTRPNNNTRKSIRIQRGPGR-AFVTIGKI.GNMRQAHC HIV-1IIIB (SEQ ID NO:6)

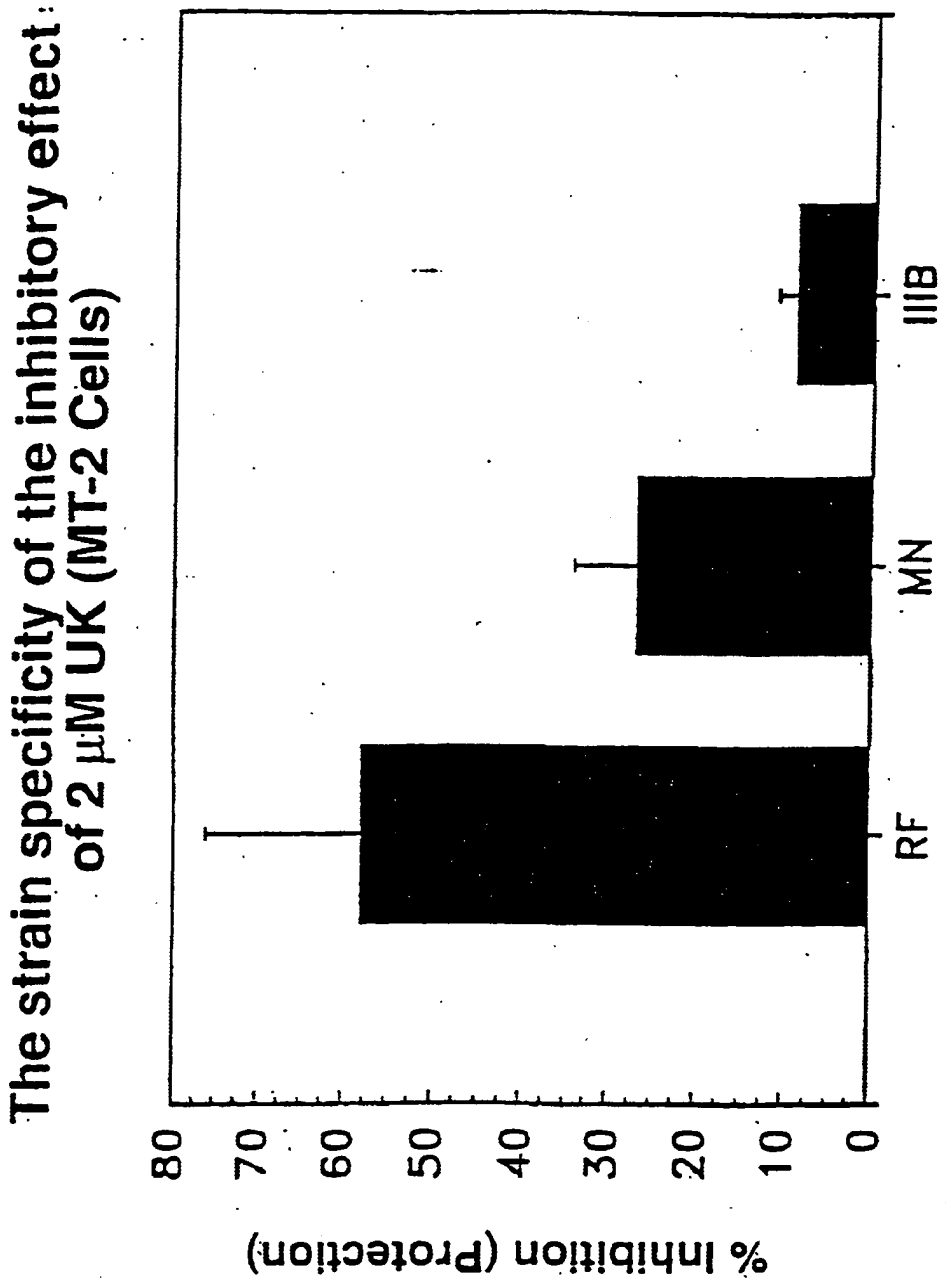

The strain specificity of the inhibitory effect of 2 μM UK (MT-2 Cells)
% Inhibition (Protection)
80
70
60
50
40
30
20
10
0
RF
MN
IIIB

FIG. 2

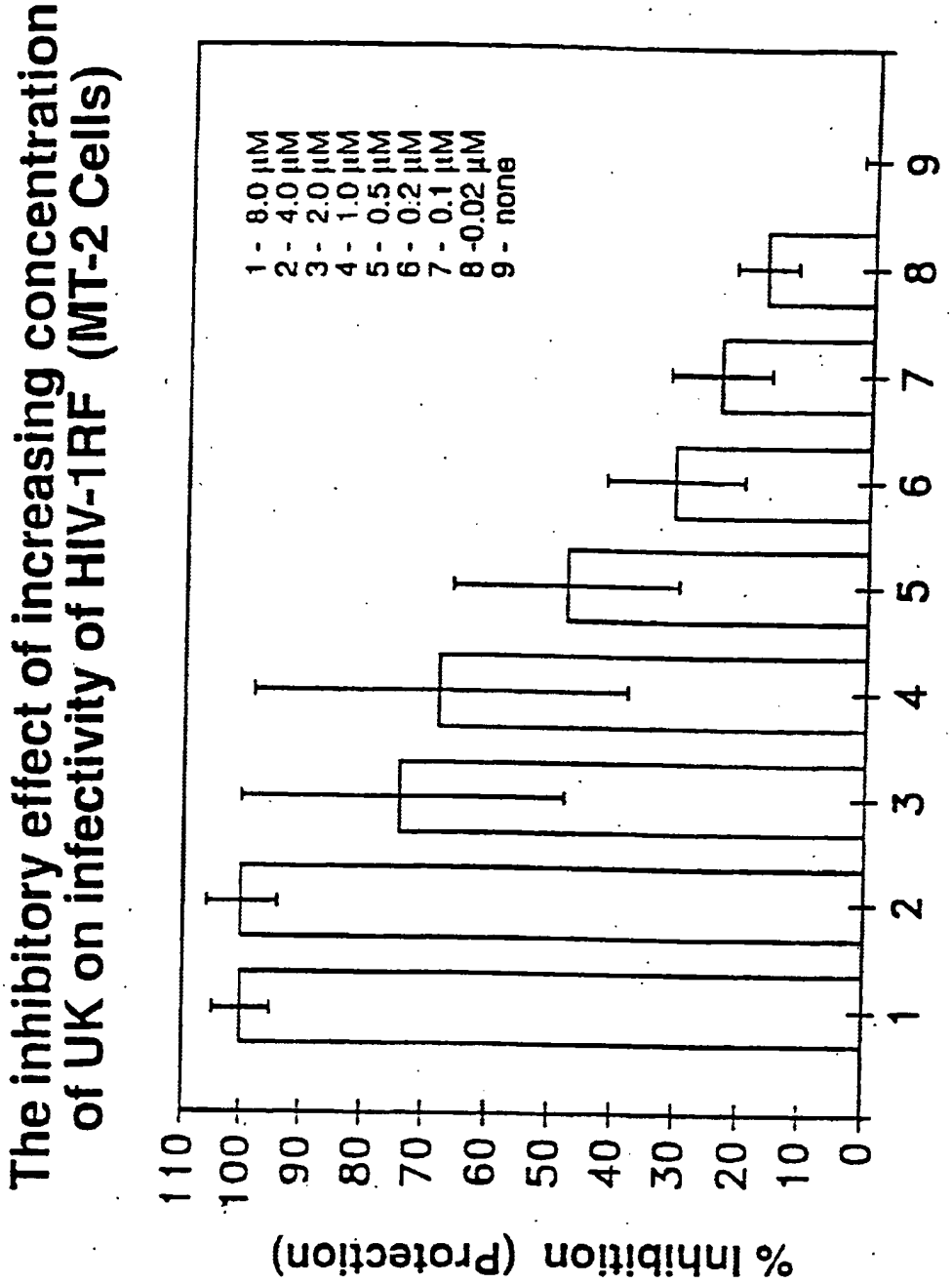

The inhibitory effect of increasing concentration of UK on infectivity of HIV-1RF (MT-2 Cells)
% Inhibition (Protection)
110
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
1 - 8.0 µM
2 - 4.0 µM
3 - 2.0 µM
4 - 1.0 µM
5 - 0.5 µM
6 - 0.2 µM
7 - 0.1 µM
8 -0.02 µM
9 - none

Fig. 3

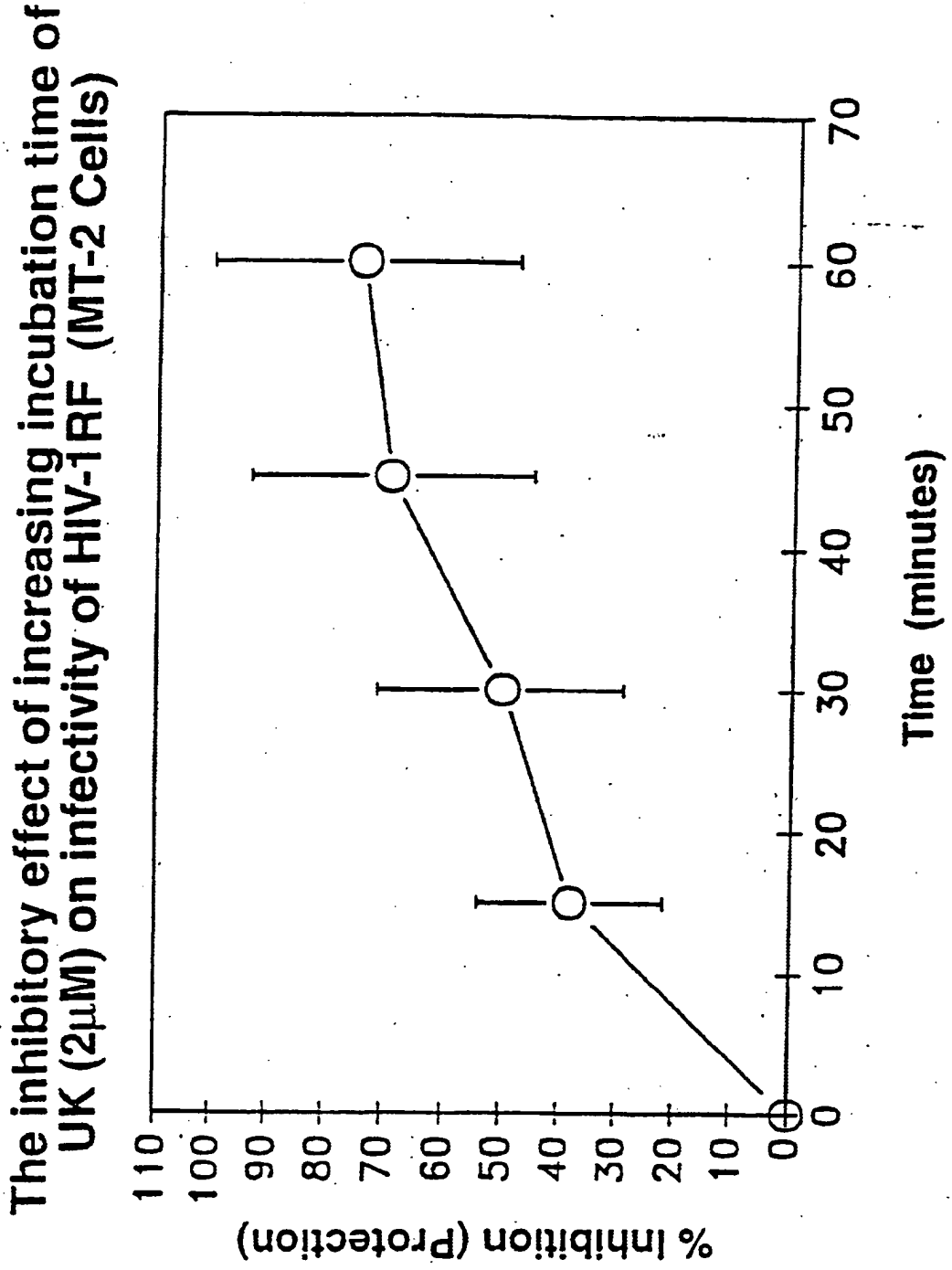
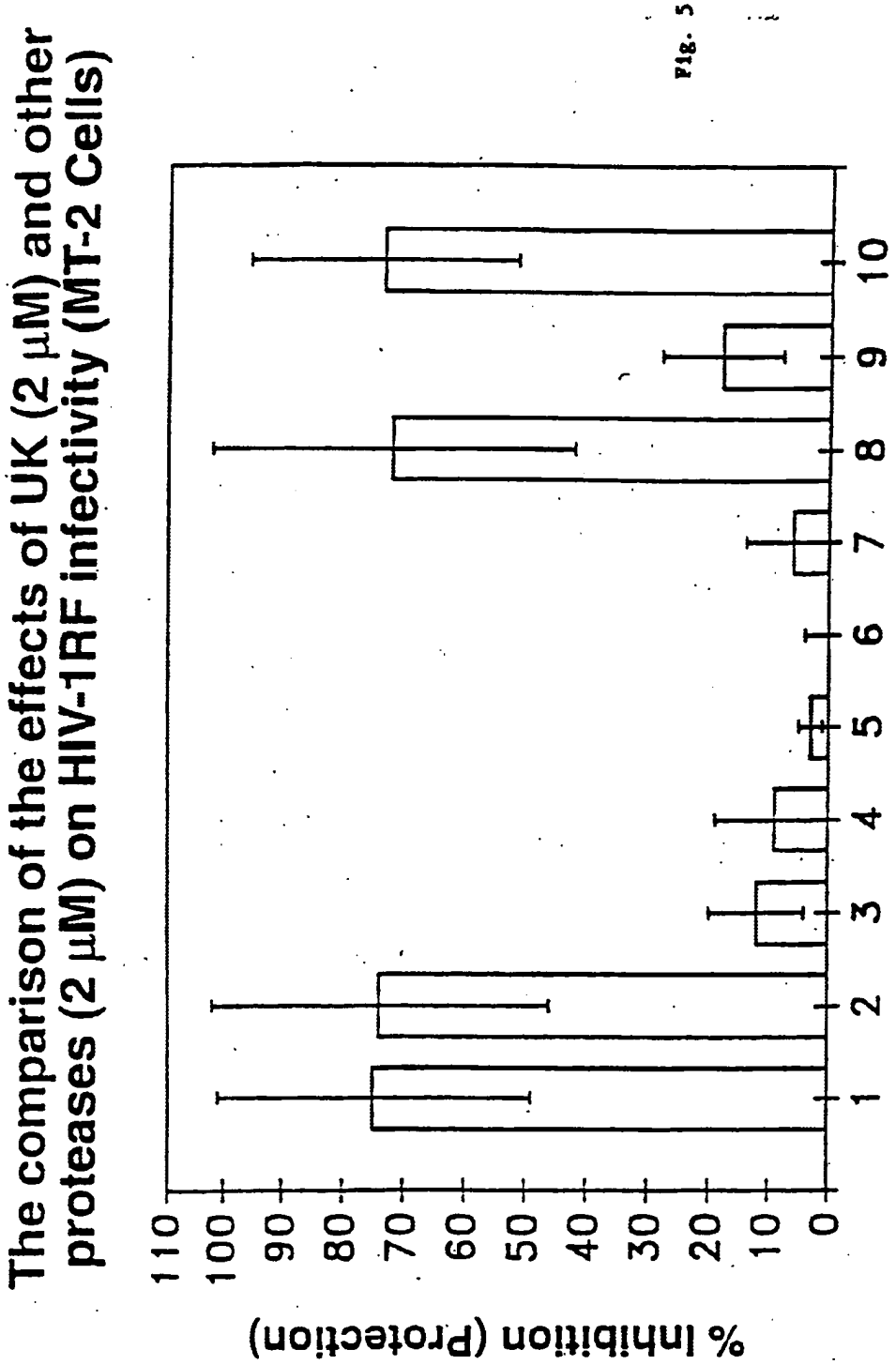

The inhibitory effect of increasing incubation time of UK (2μM) on infectivity of HIV-1RF (MT-2 Cells)
% Inhibition (Protection)
110
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
60
70
Time (minutes)

Fig. 4

Fig. 5


The comparison of the effects of UK (2 μM) and other proteases (2 μM) on HIV-1RF infectivity (MT-2 Cells)
% Inhibition (Protection)
0
10
20
30
40
50
60
70
80
90
100
110
1
2
3
4
5
6
7
8
9
10

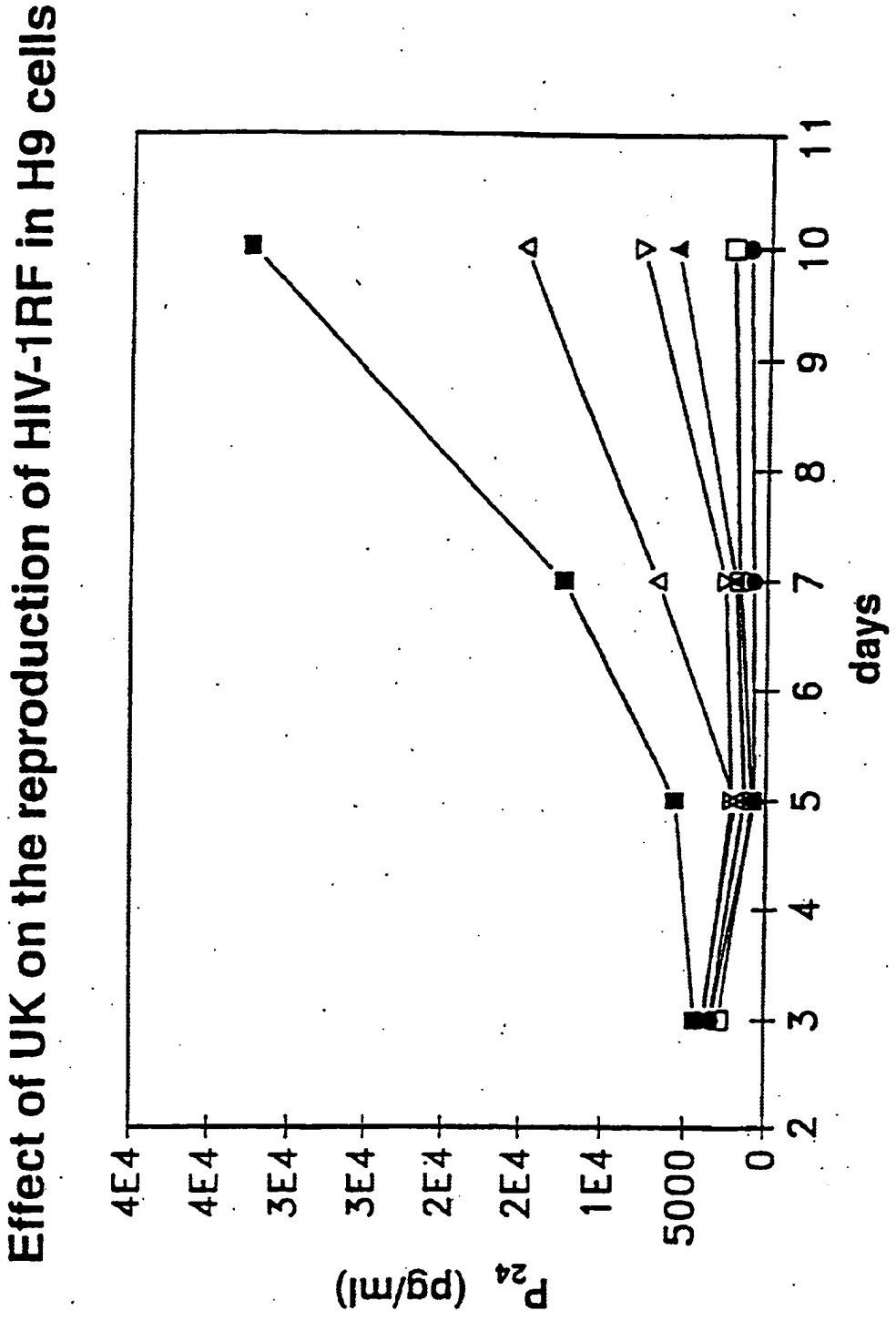

Effect of UK on the reproduction of HIV-1RF in H9 cells
P_{24} (pg/ml)
4E4
4E4
3E4
3E4
2E4
2E4
1E4
5000
0
2
3
4
5
6
7
8
9
10
11
days

Fig. 6

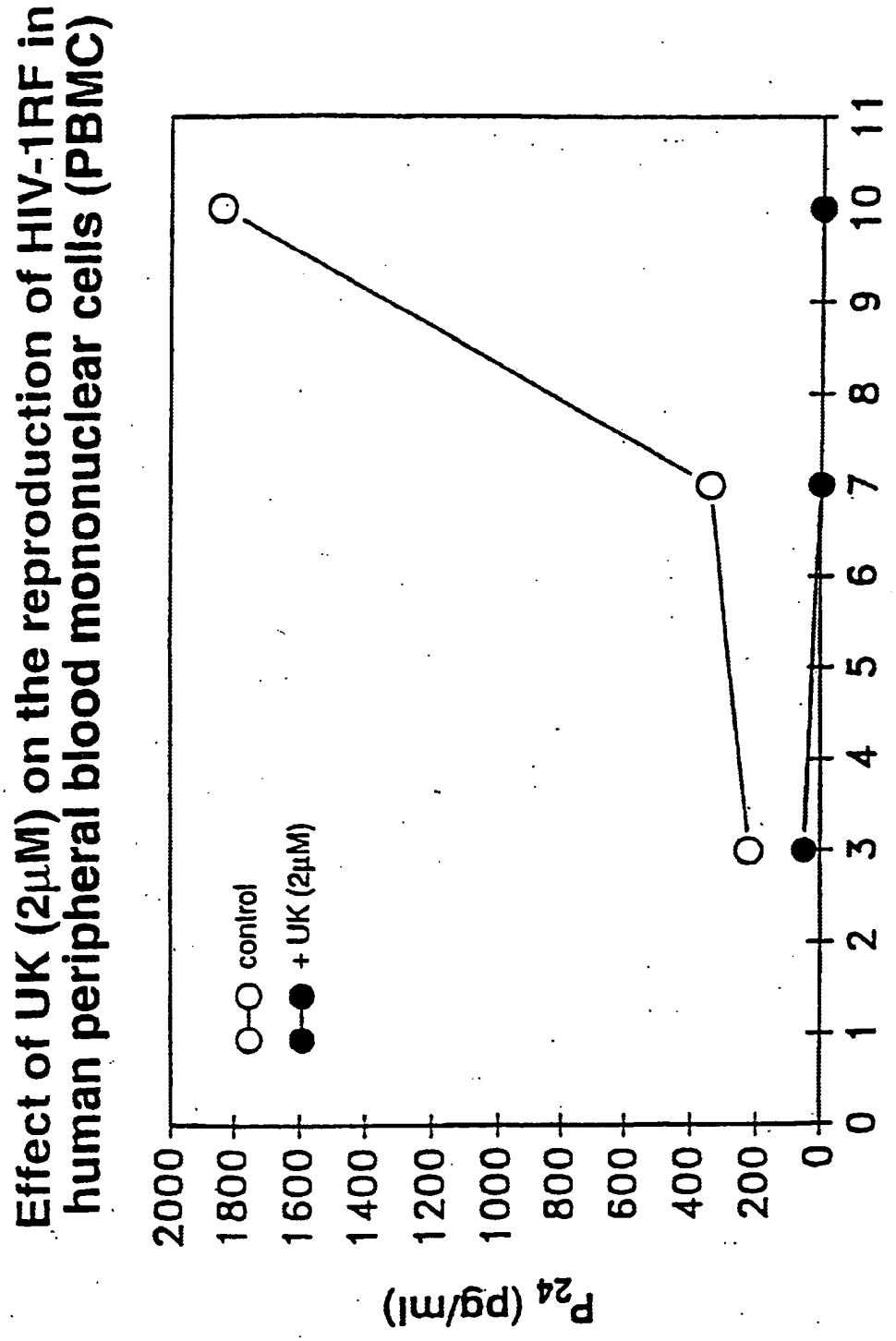

Effect of UK (2µM) on the reproduction of HIV-1RF in human peripheral blood mononuclear cells (PBMC)
P_{24} (pg/ml)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0 1 2 3 4 5 6 7 8 9 10 11
days
control
+ UK (2µM)

FIG. 7

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14093

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01N 1/02; C12N 11/00; C12N 5/00; A61K 38/46;
US CL :435/ 2, 174, 240.2; 424/94.63; 514/2, 12, 21; 530/324, 350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/2, 174, 240.2; 424/94.63; 514/2, 12, 21; 530/324, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS ONLINE, MEDLINE, BIOSIS, WPIDS
Search terms: AIDS, HIV, UROKINASE, PLASMINOGEN, INHIBIT?, TREAT?, REDUC?, INACTIV?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	Immunological Investigations, Volume 21, No. 4, issued 1992, Auci etal., "Dysregulated Proteolysis In AIDS", pages 305-319, especially pages 306-307.	1, 2, 3, 5, 6, and 9 ---- 4, 7-8, and 10-22
X ---- Y	Journal of Leukocyte Biology, Volume 52, issued September 1992, Auci et al., "Constitutive production of PAI-II and increased surface expression of GM1 ganglioside by peripheral blood monocytes from patients with AIDS: evidence of monocyte activation in vivo", pages 282-286, especially page 284.	1, 2, 3, 5, 6 and 9 ---- 4, 7, 8 and 10-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 11 JANUARY 1996	Date of mailing of the international search report 27.02.96
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer Deborah Frelse for IRENE MARX Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/14093

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	Federation of American Societies for Experimental Biology, 71st Annual Meeting, Paper No. 6046, issued 1987, Auci et al., "Serum from AIDS patients lacks the inhibitor(s) of exogenous urokinase-dependent fibrinolysis", page 1353, especially Abstract.	1, 2, 3, 5, 6 and 9 — 4, 7, 8, and 10-22
X	Clinical Research, Volume 42, No. 2, issued 1994, Handley et al., "Urokinase (uPA) cleaves gp 120 of HIV-1 and promotes viral infectivity", page 155A, especially Abstract.	1, 2, 3, 5, 6 and 9
Y	AIDS, Volume 6, No. 1, Issued 1991, Meylan et al., "HIV infectivity is not augmented by treatment with trypsin, Factor Xa or Human mast-cell tryptase", pages 128-130. See whole document.	1-22
Y	AIDS Research and Human Retroviruses, Volume 7, No. 1, issued 1991, Clements et al., "The V3 Loops of the HIV-1 and HIV-2 Surface Glycoproteins Contain Proteolytic Cleavage Sites: A Possible Function in Viral Fusion?", pages 3-16. See whole document.	1-22
Y	Nature, Volume 343, issued 18 January 1990, Stephens et al., "A chink in HIV's armour?", page 219. See entire document.	1-22
Y	FEBS Letters, Volume 248, Number 1,2, issued May 1989, Hattori et al., "Involvement of tryptase-related cellular protease(s) in human immunodeficiency virus type 1 infection", pages 48-52. See whole document.	1-22
Y	AIDS Research and Human Retroviruses, Volume 9, No. 2, issued 1993, Schulz et al., "Effect of Mutations in the V3 Loop of gp120 on Infectivity and Susceptibility to Proteolytic Cleavage, pages 159-166. See whole document.	1-22
Y	Protein Engineering, Volume 1, No. 6, issued 1987, T. J. R. Harris, "Second-generation plasminogen activators", pages 449-458. See whole document.	1-22